Is there a hybridization barrier between *Gentiana lutea* color morphs?

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In *Gentiana lutea* two varieties are described, *G. lutea* var. *aurantiaca* with orange corolla colors and *G. lutea* var. *lutea* with yellow corolla colors. Both varieties co-occur in NW Spain. However, it is not known whether a hybridization barrier exists between these *G. lutea* color varieties. If that is the case, this barrier might reflect that *G. lutea* varieties are the product of a secondary contact or that they are currently under an ongoing speciation process. We know that abiotic environmental factors are not related to flower color variation among *G. lutea* populations in NW Spain. But, pollinators cause selection on flower color in this species, if *G. lutea* is dependent on pollinators, they might be currently driving or reinforcing the existence of these two morphs. Thus, we aim to test the compatibility between flower color varieties in *G. lutea* and its dependence on pollen vectors. Within a *G. lutea* population we analyzed differences in reproductive success (number, weight, viability and germinability of seeds) depending on fertilization treatments (autogamy and xenogamy within variety and among varieties). Our results confirmed that reproductive success is higher within color varieties than among varieties, due to seed viability reduction on hybrids from different varieties, and that *G. lutea* reproductive success is strongly dependent on pollinators. Regardless of the allopatric or sympatric origin of *G. lutea* flower color, we conclude that a partial hybridization barrier exists between *G. lutea* varieties, which are at least partially driven by the dependence of *G. lutea* on pollinators.

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Abstract

In *Gentiana lutea* two varieties are described, *G. lutea* var. *aurantiaca* with orange corolla colors and *G. lutea* var. *lutea* with yellow corolla colors. Both varieties co-occur in NW Spain. However, it is not known whether a hybridization barrier exists between these *G. lutea* color varieties. If that is the case, this barrier might reflect that *G. lutea* varieties are the product of a secondary contact or that they are currently under an ongoing speciation process. We know that abiotic environmental factors are not related to flower color variation among *G. lutea* populations in NW Spain. But, pollinators cause selection on flower color in this species, if *G. lutea* is dependent on pollinators, they might be currently driving or reinforcing the existence of these two morphs. Thus, we aim to test the compatibility between flower color varieties in *G. lutea* and its dependence on pollen vectors. Within a *G. lutea* population we analyzed differences in reproductive success (number, weight, viability and germinability of seeds) depending on fertilization treatments (autogamy and xenogamy within variety and among varieties). Our results confirmed that reproductive success is higher within color varieties than among varieties, due to seed viability reduction on hybrids from different varieties, and that *G.*

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Keywords Pollen vectors, *Gentiana lutea*, flower color morphs, self-incompatibility, hybridization barrier, outcrossing success.

Introduction

Pollen vectors can drive plant evolution and diversification throughout their selection on floral traits (Darwin, 1859; Darwin, 1862; Thompson, 1994; Barrett & Harder, 1996; Charlesworth, 2006). In particular, animal pollinators exert selective pressures on plant traits through natural selection (Stebbins, 1970) and many flowering plants rely on those pollen vectors to reproduce. Species widely differ in their dependence on pollen vectors for seed production, from complete autogamy to xenogamy, requiring cross-pollination for successful reproduction (Darwin, 1877; Axelrod, 1960). Most angiosperms need vectors for pollen transference between plants, which are mainly insects but can also be other animals and to a lesser extent wind or water (Harder & Barrett, 1996; Ackerman, 2000; Ollerton, Winfree & Tarrant, 2011). Different floral strategies were developed to attract these animals (Ghazoul, 2006), which may affect plant fitness to a large degree (Waser, 1983; Conner & Rush, 1996). This plant-pollinator relationship promotes evolution of species involved in such an interaction. Thus, the degree of dependence on animal pollinators might affect the strength of selection and therefore the likelihood of species diversification.

Flower color variation in polymorphic species may originate from different selective pressures exerted by pollinators, which favor isolation between different color morphs and

cause sympatric diversification (Gegear & Burns, 2007). Thus, pollinator behavior may favor one of the color morphs, with individuals visiting one color morph more frequently than a different flower color morph (Hopkins & Rausher, 2012). Additionally, many plant species show floral divergences due to geographic isolation suffered by climatic changes during Quaternary period (Gómez & Lunt, 2007; Vargas et al., 2009; Martín-Bravo et al., 2010; Fernández-Mazuecos &Vargas, 2011; Blanco-Pastor, Vargas & Pfeil, 2012; Blanco-Pastor & Vargas, 2013; Fernández-Mazuecos et al., 2013). Thus, a secondary contact between flower color varieties that remained geographical isolated after a long time, could explain reproductive incompatibility between floral color morphs. Despite the origin of the phenotypic floral variation, diversification between flower color varieties may be reinforced by pollinator preferences (Campbell, Alarcon & Wu, 2003).

Gentiana lutea L. shows flower color variation from orange to yellow both, within and among populations (Sobral et al., 2015) at the western extreme of the distribution range. Two different varieties are described for *Gentiana lutea* L. depending on flower color: *Gentiana lutea* L. var. *aurantiaca* (M. Laínz) showing orange corolla colors and *Gentiana lutea* L. var. *lutea* showing yellow corollas (Laínz, 1982; Renobales, 2003). *Gentiana lutea*'s flower color is a trait with a genetic basis (Zhu et al., 2002; Zhu et al., 2003) and there are genetic differences among populations (González-López et al., 2014). We know that flower color variation among populations in this species across NW Spain, is not related to abiotic environmental factors such as elevation, temperature, radiation and rainfall (Veiga et al., 'in press'). Additionally, we know that pollinators exert selective pressures on *G. lutea* flower color (Veiga et al., 2015) and play a role in flower color differentiation among *G. lutea* populations (Sobral et al., 2015). But, it is not completely understood the degree of dependence of *G. lutea* on pollen vectors to successfully reproduce. Rossi (2012) described *G. lutea* as a partial self-compatible species. However, others cite *G. lutea* as a self-incompatible

75 species, which may need animal pollinators to reproduce (Hegi, 1927; Kèry, Matthies &
76 Spillmann, 2000; Kozuharova & Anchev, 2006; González-López et al., 2014).

77 The aim of this study is to test if there is some degree of incompatibility between *Gentiana*
78 *lutea* color varieties due to a partial hybridization barrier, which might be caused or reinforced
79 by selective pressures exerted by pollinators on *G. lutea* flower color. If a hybridization barrier
80 exists between *G. lutea* color morphs we might expect crossings among varieties to have a
81 lower reproductive success (Hauser, Jørgensen & Østergård, 1998; Hauser, Shaw &
82 Østergård, 1998). Additionally, if *G. lutea* color morphs are under a diversification process
83 driven by selective pressures exerted by pollinators (Veiga et al., 2015; Sobral et al., 2015),
84 we would expect *G. lutea* to strongly depend on the pollinating vectors exerting this selection.
85 Thus, *G. lutea* crossings among different individuals would have higher reproductive success
86 than crossings within-individual plants. In order to investigate our hypothesis, we proposed
87 the following questions; *(i)* Are there any differences in reproductive success (seed number,
88 seed weight, seed viability and seed germinability) between within-color variety crossings
89 and among-color variety crossings? (ii) Are there any differences in reproductive success
90 between autogamic seeds and seeds coming from among individual crossings?

91

92 ## Materials & Methods

93 *Gentiana lutea* L. (Gentianaceae) is a herbaceous perennial plant distributed along the Central
94 and Southern European mountains typically growing on livestock grazing grasslands and
95 hillsides from montane to sub-alpine habitats, approximately from 800 to 2.500 m a.s.l.
96 (Hesse, Rees & Müller-Schärer, 2007; Anchisi et al., 2010). This long-lived species presents a
97 rhizome, which develops one (rarely two or three) unbranched stout stem with basal leaves,
98 approximately growing up to 200 cm tall (Renobales, 2012). Flowering occurs in summer

(June-July) when the fertile stems show flowers, grouped in pseudo-whorls, which bloom spirally; and the inflorescence develops in succession from apex to base (Kozuharova, 1994). There are two different varieties that differ in flower color: *Gentiana lutea* L. var. *aurantiaca* (M. Laínz) with orange flowers and *Gentiana lutea* var. *lutea* L. with yellow flowers (Laínz, 1982; Renobales, 2003). *Gentiana lutea* flowers show rotate corollas with lobes (Renobales, 2012), which facilitate the access of pollinators to the nectaries; and are visited by at least 11 families of insects, belonging to four orders (Rossi et al., 2014). The main flower visitors of *G. lutea* plants at the Cantabrian Mountains are bumblebees, followed by cuckoo bumblebees and honeybees (Sobral et al., 2015). Fruits hold many flattened, elliptic and winged seeds disseminated through anemochory (Struwe & Albert, 2002).

We carried out experimental manipulations at one *G. lutea* population in León, Spain (43º 03' N; 6º 04' W; 1600 m a.s.l.) in July 2012. For field experiments, we received field permit from Environmental Territorial Service from León, Territorial Delegation of Government of Spain, Regional Government of Castilla and León (Identifier:12_LE_325_RNA_PuebladeLilio_INV; Reference: 06.01.013.016/ROT/abp; File number: AEN/LE/103/12). Note that in this study, we will distinguish two different flower color classes (orange and yellow). Both varieties do not differ in their UV reflectance but they do differ in their visible reflectance (see Veiga et al., 2015); thus, we use visible reflectance to indentify color varieties. We haphazardly chose five flowers on each of 25 orange-flowered plants and 25 yellow-flowered plants. Flower buds, except the control group, were bagged with tulle before flower opening and until fruit formation in order to avoid contact with pollen vectors.

We created the following treatments: (1) Control group (C), in which we applied no treatment, so natural pollination occurred; (2) Spontaneous autogamy (Sa), in which pollen was not applied manually and only pollen from the same flower may spontaneously arrive to the ovary; (3) Facilitated autogamy (Fa), in which we applied pollen manually from the flower's

own anthers; (4) Facilitated xenogamy within varieties (Fxw), in which we emasculated flowers by cutting the stamens before pollen release in order to prevent the entry of flower's own pollen, and applied pollen manually from other plants of the same flower color; (5) Facilitated xenogamy among varieties (Fxa), in which we emasculated the flowers and applied pollen manually from plants with different flower color.

Reproductive success can be quantified as the number of fertile descendants produced by an individual throughout its life. It is not feasible to quantify reproductive success in long-lived species in these terms, so seed production is considered a good estimate (see the review of Kingsolver et al., 2001). Other measures of reproductive success are seed viability and seed germinability. We used four measures of reproductive success: number of seeds, weight of seeds (mg), rate of viable seeds (seed viability) and germination rate (seed germinability). Due to manipulations some bags were opened, thus plants with the five treatments were down to 26. 130 ripped fruits were collected before opening, being careful to ensure that the seeds were fully formed. On each fruit we measured seed weight (mg) and counted the number of seeds and the number of ovules not developed; from the sum of non-fertilized ovules and the seeds we obtained the total number of ovules. Total number of ovules did not vary among treatments (data not shown), thus we used the absolute number of seeds produced in each fruit, instead of calculating the seed production relative to the ovules on each fruit.

We also measured seed germinability and seed viability. We haphazardly chose up to 20 seeds in each fruit and distributed them on filter paper in petri plates. Seed number was very low in the autogamy treatments; thus, seeds from spontaneous autogamy (Sa) and facilitated autogamy (Fa) treatments were grouped. In total, we analyzed viability and germination rate of 1,800 seeds (10 plates per treatment and between 30 and 50 seeds per plate). Germination was induced with gibberellic acid 100 mg/L at 24 h of darkness and constant temperature of 23 °C (Bell et al., 1995). The state of germination and wetting of the plates were controlled on

149 alternate days; the filter paper was removed every 2-3 days to reduce fungal infection. Seeds
150 with a radicle of at least 2 mm were considered germinated. Seed germinability is the
151 percentage of germinated seeds. We controlled germination rate for a period of 45 days. After
152 45 days we tested seed viability by crushing the seeds with the tip of the tweezers. Soft and
153 dark seeds were not considered viable and harsh and light-colored seeds were considered
154 viable (as Hesse, Rees & Müller-Schärer, 2007). These seeds along with the germinated seeds
155 were considered the total number of viable seeds.

156 We calculated the Self-Compatibility Index (SCI) to describe the *G. lutea* breeding system (as
157 Lloyd & Schoen, 1992). SCI is assessed as the average seed set for facilitated autogamy (Fa)
158 divided by the average seed set for facilitated xenogamy (Fxw or Fxa) and gives information
159 about the self-compatibility of the species. SCI values range from 0 to 1.5 and a species is
160 considered self-incompatible when its values are between 0 and 0.75 (Lloyd & Schoen,
161 1992).

162 To assess the occurrence of self-fertilization, we calculated the Auto-Fertility Index (AFI)
163 dividing the seed set for spontaneous autogamy (Sa) by the seed set for facilitated xenogamy
164 within varieties (Fxw or Fxa) (Lloyd & Schoen, 1992). AFI gives information about the
165 autonomous autogamy degree of the species.

166 In order to analyze the differences in seed number and seed weight among treatments we used
167 a generalized linear mixed model (GzLMM); the fixed factors were the treatment (five
168 categories: C “Control group”, Sa “Spontaneous autogamy”, Fa “Facilitated autogamy”, Fxw
169 “Facilitated xenogamy within varieties”, Fxa “Facilitated xenogamy among varieties”), the
170 maternal flower color (color variety) and the interaction between color variety and treatment.
171 Plant individual was a random factor nested within maternal flower color (color variety).

172 To test for differences in seed viability and germinability depending on treatment, we used a
173 generalized linear model (GzLM) for each response variable. Treatment was a fixed factor

(with four categories, since the seeds of spontaneous and facilitated autogamy treatments were joint to have a sufficient sample). Note that first, we performed a generalized linear mixed model (GzLMM), where treatment was a fixed factor and plate number was a random factor nested within treatment, but it did not converge. Additionally, we could not test if the germination rate varied between colors or individuals because of insufficient sample size. Error distribution and link function were selected to minimize the AIC_C: number of seeds was fitted to a Poisson distribution and a logarithmic link function; whereas weight of seeds was fitted to a Linear distribution and identity link function. Both seed viability and seed germinability were adjusted to a Binomial distribution and probit link function. Analyses were performed with SPSS software (IBM SPSS Statistics for Windows, Version 20.0, IBM Corp.,Somers, NY).

Results

The most successful reproductive mechanism for *Gentiana lutea* was found to be cross-pollination between individuals within the same color variety. Conversely, cross-pollination among flower color varieties reduced plant reproductive success. In addition, we found that *G. lutea* depends on pollinators to reproduce.

Number of seeds, seed weight and germinability were similar for within-color variety crossings than for among-color variety crossings (Table 1; Fig. 1; Fig. 2). However, seeds from crossings within varieties present higher viability than seeds from among variety crossings (Fig. 2). Thus, seed viability decreases when pollen donor and receptor belong to different color varieties (Fig. 2). This fact may imply that seed viability decrease is an important component of the partial hybridization barrier between *G. lutea* color morphs.

Fruits under autogamy treatments produced fewer and lighter seeds than fruits from inter-

individual crossings. In addition, the effect of treatments on seed weight was the same for both color varieties (no significant color variety * treatment interaction: $p > 0.05$; Table 1; Fig. 1); although we found that under natural pollination, orange-flowering individuals produced more seeds per fruit than yellow-flowering individuals (significant simple effect of flower color: $p < 0.001$; Table 1; Fig. 1). On the contrary, within the spontaneous autogamy treatment, yellow-flowering individuals produced more seeds per fruit ($p < 0.001$; Table 1; Fig. 1). Additionally, we found significant differences among treatments for both, seed viability and seed germinability ($p < 0.001$; Table 1; Fig. 2). Note that both xenogamy treatments resulted in greater seed germinability than the autogamy treatments and control group (Fig. 2).

The Self-compatibility index scored 0.0687. SCI ranges from 0 to 1.5, with values of SCI under 0.75 meaning that the species is self-incompatible. Thus, *Gentiana lutea* is a self-incompatible species which relies in cross-pollination to successfully reproduce. Auto-Fertilization Index (AFI) scored 0.0695 meaning that *G. lutea* is a non-autogamous species.

Discussion

The most advantageous reproductive mechanism for *Gentiana lutea* L. is cross-pollination between the same color morphs. Our results suggest that a partial hybridization barrier exists between *G. lutea* flower color morphs, likely due to a past or ongoing divergence between *G. lutea* varieties. Regardless of the origin of both varieties, it seems that the differentiation is driven or reinforced by the strong dependency of *G. lutea* on pollen vectors which exert selective pressures on flower color (Veiga et al., 2015; Sobral et al., 2015).

It is well known that flower color and other floral traits (aroma, shape, flower sap content) play an essential role on plant reproduction, because they lead animal preferences (Tepedino,

1979; Quattrocchio et al., 1999). Specific sets of floral traits (pollination syndromes) are often associated with particular groups of pollinators. This close animal-plant interaction among species may drive floral evolution (Fenster et al., 2004; Hoballah et al., 2007). Angiosperm diversification may derive from reproductive isolation due to changes in pollinator habitat composition (Bradshaw & Schemske, 2003; Streisfeld & Kohn, 2007; Hoballah et al., 2007; Dauber et al., 2010). A speciation process among flower color varieties of the same species can occur (Straw, 1955; Waser, 1998; Gegear & Burns, 2007) and may be driven by differences in pollinator community, which show higher preference for one morph as many studies suggest (Dronamraju, 1960; Quattrocchio et al., 1999; Hopkins & Rausher, 2012). We know that *Gentiana lutea*'s pollinators show different color preferences among populations at the Cantabrian Mountains (Spain), partially due to differences on the pollinator spectrum within each population and specific flower color preferences from each pollinator species (Sobral et al., 2015). Sympatric speciation could originate due to selective pressures exerted by the pollinators (Quattrocchio et al., 1999; Hopkins & Rausher, 2011) that facilitate isolation between flower color varieties when cross-pollinations are avoided or reduced (Hopkins & Rausher, 2012).

Floral divergence is not always sufficient to produce isolation between morphs and consequent speciation (Fernández-Mazuecos &Vargas, 2011). The geographic isolation produced by the Quaternary climatic changes, which can be accompanied by differences in the pollinator spectra from isolated populations, has been identified as the main cause of divergence in several mountane plant species (Hewitt, 2000; Thompson, 2005; Martín-Bravo et al., 2010; Alarcón et al., 2012; Blanco-Pastor &Vargas, 2013; Fernández-Mazuecos et al., 2013). A subsequent secondary contact between both color morphs in *Gentiana lutea* L. could generate a similar situation to that actually shows our study population and surrounding area, whether is a recent or an old contact, it seems that the maintenance of different color morphs

247 is reinforced by the fitness reduction on hybrids and the pollinator behavior, as our results
248 confirmed (Table 1; Fig. 1; Fig. 2).

249 The Self-Compatibility Index obtained here suggests that *Gentiana lutea* L. is a self-
250 incompatible species (Lloyd & Schoen, 1992). Therefore, this species relies on pollen vectors
251 for a successful reproduction, as in the case of the most flowering-plant species (Axelrod,
252 1960; Tepedino, 1979). The Self-Compatibility Index (SCI=0.068) results were similar to the
253 Auto-Fertilization Index (AFI= 0.069). Note that SCI is the ratio between the average seeds
254 produced in facilitated autogamy treatment and the average seeds produced in facilitated
255 xenogamy, whereas AFI is assessed from the ratio between seeds set from spontaneous
256 autogamy and facilitated xenogamy treatment. Similar values of the two indexes imply that
257 the number of seeds produced when a flower's own pollen arrives both, naturally and
258 manually is similar. This fact may suggest that self-incompatibility in this species is not
259 caused by a physical barrier separating pollen from the same flower, but is caused by some
260 pre- zygotic barrier mechanism in which pollen from same flower is not fertilizing the ovules,
261 or by some post- zygotic barrier that may produce a lower quality and number of autogamic
262 seeds (Charlesworth & Charlesworth, 1987; Hopkins, 2013).

263 It is an interesting fact that seed number and weight, and to a lesser extent seed germinability,
264 from natural pollination is intermediate between autogamy treatments and xenogamy
265 crossings (see Fig. 1; Fig. 2). This result suggests that pollen vectors carry to a plant, in
266 natural conditions, both pollen from that plant and pollen from different plants. Therefore,
267 those animal pollinators that have higher mobility between plants and lower mobility among
268 flowers within plants would pollinate more successfully (Dauber et al., 2010; Rossi et al.,
269 2014).

270 On the other hand, yellow-flowering plants set a greater number of seeds when spontaneous
271 self-pollination occurs, which might suggest that the self-incompatibility degree in this

species varies among varieties and is actually higher for orange-flowering individuals (Fig. 1). This implies that the Auto-Fertilization Index might vary among color morphs. Additionally, we found differences in seed number within the natural pollination treatment, in which orange flowers set a greater number of seeds. We know that pollinator assemblage shows preferences for yellowness within our study population, in which yellow-flowering individuals have a greater total seed set (Veiga et al., 2015). However, our results suggest that although orange-flowering plants have lower fitness than yellow ones, they produce more seeds per fruit (see Table1; Fig. 1).

We know that pollinators exert selective pressures on *Gentiana lutea* L. flower color (Veiga et al., 2015) and that these selective pressures drive flower color differentiation in this species (Sobral et al., 2015). Additionally, we know that abiotic factors such as temperature, radiation, elevation and rainfall are not related to flower color variation among *G. lutea* populations (Veiga et al., 'in press'). With the available information, it is not clear whether we are dealing with an ecological speciation process in which the original color differentiation is due to an allopatric or sympatric process; but our results bring to light the existence of phenotypic differentiation and a lower fitness in hybrids. Regardless of the origin of the divergence, it seems that today a divergence process is ongoing among *G. lutea* color morphs driven by selection on flower color exerted by pollinators.

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470 **Table 1: Effect of different treatments of pollination on female reproductive success**

471 **(number of seeds, seeds weight, seed viability and seed germinability).**

472 We marked in bold the statistically significant factors ($P < 0.05$).

Response Variable	N	Factors	Variance	S.E.	Wald Chi-Square	d.f.	*P*
Seed number	130	*Random effect*					
		Plant (Color variety)	0.050	0.016			
		Fixed effects					
		Treatment			504.389	4	**0.000**
		Color morph			0.001	1	0.971
		Color morph*Treatment			23.222	4	**0.000**
Seed weight	108	*Random effect*					
		Plant (Color variety)	6.748	3.112			
		Fixed effects					
		Treatment			44.347	4	**0.003**
		Color morph			0.004	1	0.949
		Color morph *Treatment			0.177	4	0.950
Seed viability	1,800	*Fixed effects*					
		Treatment			30.906	3	**0.000**
Seed germinability	1,800	*Fixed effects*					
		Treatment			32.966	3	**0.000**

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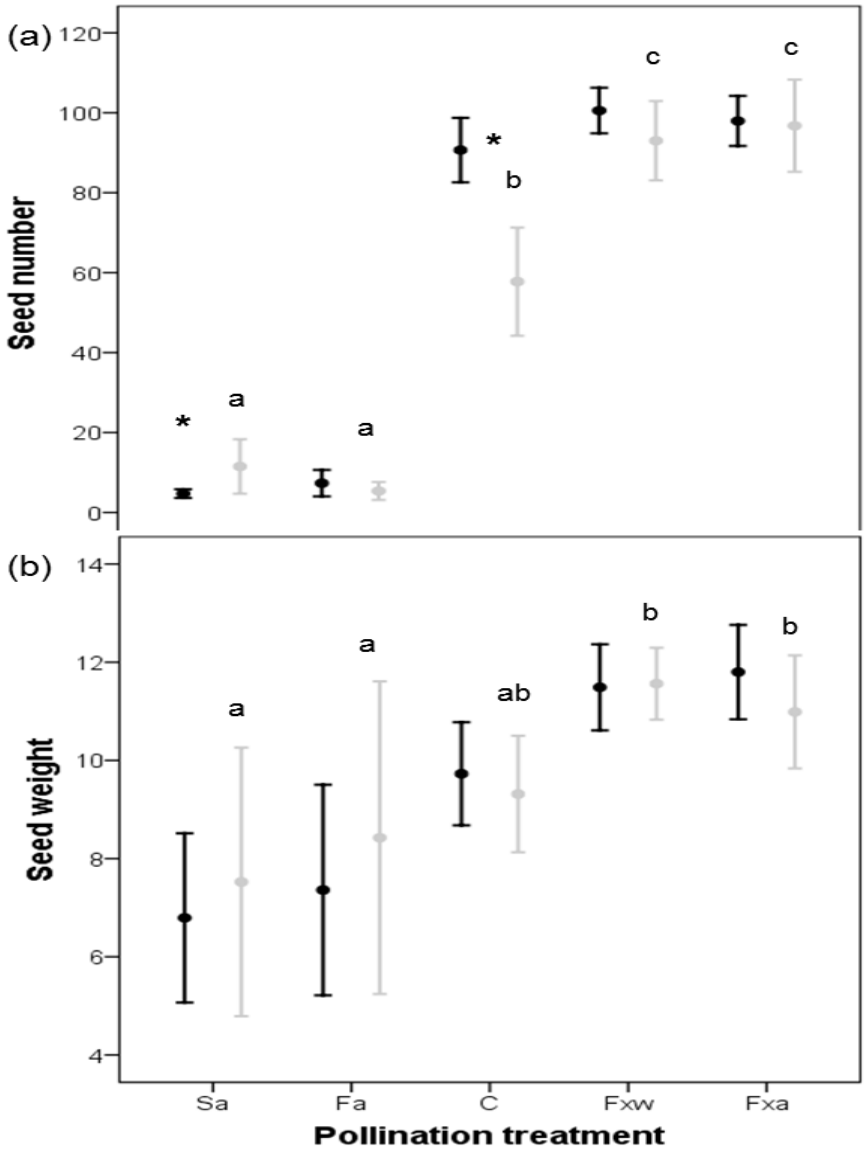

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476 **Figure 1: Reproductive success assessed by seed number (A) and seed weight in mg (B),**
477 **in function of different pollination treatments.**

478 Pollination treatments were: Spontaneous autogamy (Sa); Facilitated autogamy (Fa); Control
479 group (C), Facilitated xenogamy within varieties (Fxw) and Facilitated xenogamy among va-
480 rieties (Fxa). Significant statistical differences between treatments are marked with different
481 letters, and significant statistical differences between color varieties (orange: black-colored
482 bars; yellow: grey-colored bars) are marked with an asterisk. Bars show the Standard Error
483 (S.E.).

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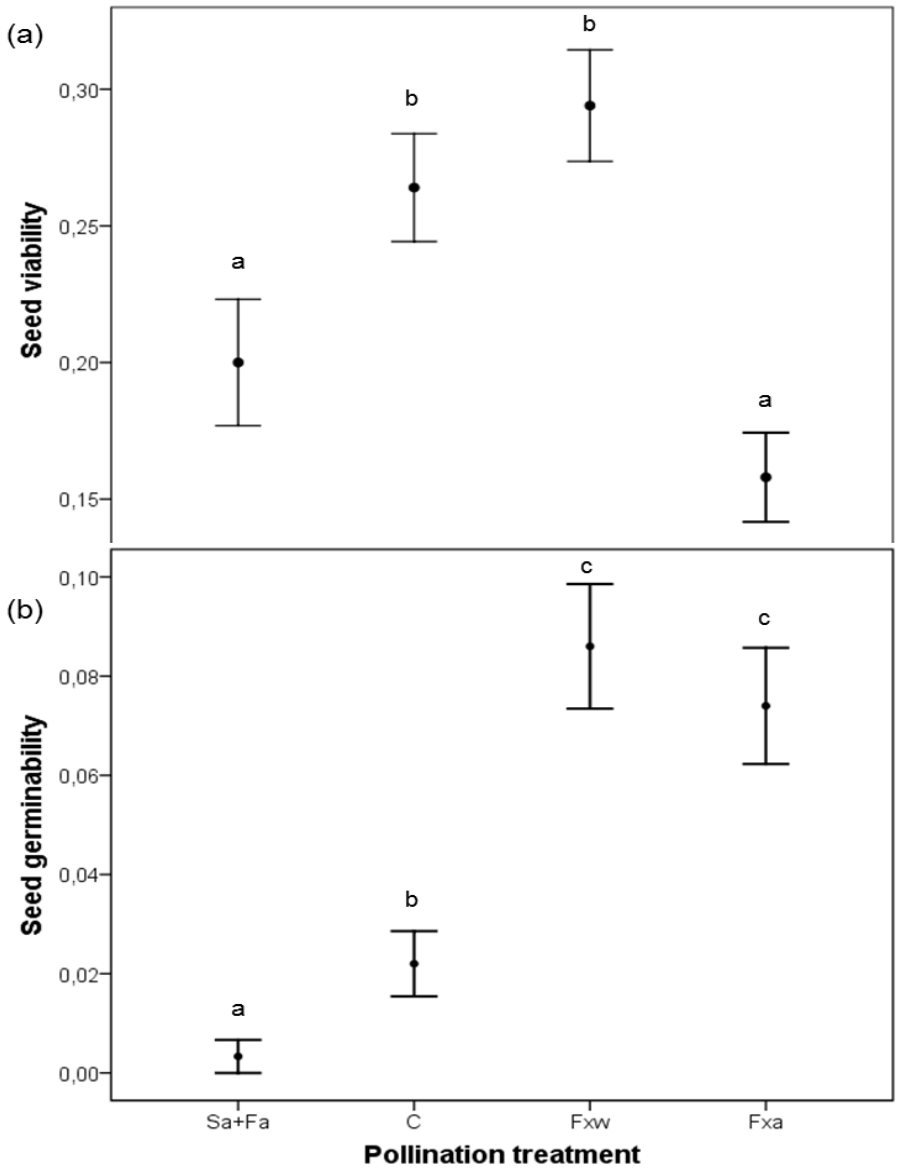

485

486 **Figure 2: Reproductive success assessed by seed viability (A) and seed germinability (B),**

487 **in function of different pollination treatments.**

488 Pollination treatments were the following: Spontaneous autogamy (Sa) + Facilitated autoga-

489 my (Fa); Control group (C), Facilitated xenogamy within varieties (Fxw) and Facilitated xe-

490 nogamy among varieties (Fxa). The statistical significant differences between treatments are

491 marked with different letters. Bars show the Standard Error (S.E.).